Conformation of Proteins and Polypeptides

II. OPTICAL ROTATORY DISPERSION AND CONFORMATION OF THE MILK PROTEINS AND OTHER PROTEINS IN ORGANIC SOLVENTS

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The study of the optical rotatory properties of synthetic polypeptides in organic solvents has contributed considerably to our understanding of the structural organization of globular proteins. However, in contrast to the extensive literature which has accumulated in the past decade on the various polyamino acids (1–3), relatively little work has been done on actual proteins in organic solvents. Weber and Tanford (4) have studied the hydrodynamic behavior and the rotatory dispersion properties of ribonuclease in 2-chloroethanol and 2-chloroethanol-water mixtures. The conversion of the native structure of the protein to a more helical structure in chloroethanol-rich solutions has been interpreted by these authors in terms of weakening and destruction of hydrophobic regions in the native protein fold. Sage and Singer (5) have found that the structural alterations in ethylene glycol lead to the normalization of the titration behavior of the three abnormally ionizing tyrosyl residues in this enzyme without any significant alterations in the helical content or irreversible loss of catalytic activity. Presumably, the abnormal tyrosyls are buried in nonhelical hydrophobic regions of ribonuclease. The optical rotatory and hydrodynamic properties of β-lactoglobulin in mixed solvents, containing up to 80% nonaqueous component, have been studied by Tanford *et al.* (6–8). These studies and studies by others in mixed solvents have led to the hypothesis that hydrophobic forces (9–13) play an important stabilizing role in the maintenance of the native structure of proteins as well as nucleic acids (14–16) in aqueous media. Recently, Hamaguchi *et al.* (17–19) have published a detailed study on the solvent denaturation of lysozyme, employing a variety of organic solvents as denaturing agents. The effects of 2-chloroethanol on the rotatory properties of a number of proteins have been reported by Imahori, Klemperer, and Doty (20) (see also References 1 and 2) and very recently by Shechter and Blout (21, 22). These are the only further studies which could serve to augment the findings of the accompanying paper (23) which deals with the extension of the solvent perturbation method of difference spectroscopy to the study of proteins and polypeptides in organic solvents. The need for auxiliary information prompted us to examine the optical rotatory properties of the seven proteins employed in the above paper. The denaturing solvents used in the present work are ethylene glycol, acidic methanol, 2-chloroethanol, 8 M urea, and, in some cases, formic acid. For comparison, we also report the rotatory properties of these proteins in aqueous media.

EXPERIMENTAL PROCEDURE[1]

The protein samples and the preparation of protein solutions inorganic solvents have been described (23). Protein concentrations were determined gravimetrically and spectrophotometrically with extinction coefficients taken from the literature or determined in separate experiments. In most cases, both aqueous and organic solutions of proteins were prepared at the same time, with the aqueous solutions serving as a check on the concentration of the organic solutions. Optical density measurements were routinely made in a Zeiss model PMQ II spectrophotometer against appropriate solvent blanks. For these measurements both aqueous and organic stock solutions were diluted volumetrically by a factor of 10 to 20, with acidic aqueous solutions or phosphate buffer, pH 6.8, as diluent. Exposure of the proteins to the organic solvents employed was found to have little or no effect on their absorbances in aqueous media. As a result, it was not necessary to make any optical density correction in cases in which the solutions had been clarified by centrifugation (*e.g.* acid-free ethylene glycol solutions) or in duplicate experiments, in which no parallel measurements were made on aqueous solutions. Urea solutions were prepared in volumetric flasks, with solid urea and aqueous protein solutions of known concentration and ionic strength. pH measurements were made with a Beckman model G pH meter.

Optical rotatory dispersion measurements were made in the 312- to 578-mμ wave length region, in a Rudolph model 200S spectropolarimeter. Both mercury and xenon arc lamps were used as sources of radiation. Demountable 2-dm polarimeter tubes with quartz windows were used throughout this work. Measurements were carried out in a room maintained at 25 ± 1°. In most cases, water was circulated from a 25° bath throughout the outer jacket of the cell compartment of the spectropolarimeter. Rotatory dispersion measurements were usually made within 1 to 6 hours after the preparation of protein solutions. The concentrations of the protein solutions employed were beween 0.3 to 0.5 g/100 ml. The optical rotation of most proteins was largely independent of time (only in the case of salt-free lysozyme in ethylene glycol was the dispersion behavior slightly time-dependent). As a consequence, measurements on lysozyme in glycol media were continued for 48 hours.

[1] Mention of specific firms and products does not imply endorsement by the United States Department of Agriculture to the possible exclusion of others not mentioned.

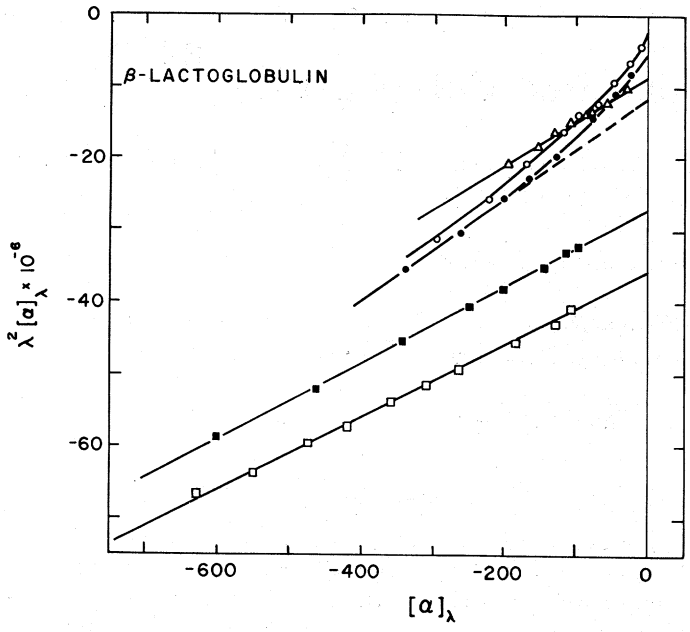

FIG. 1. Modified Lowry plot of the rotatory dispersion data of β-lactoglobulin in aqueous and organic solvents. △, water, Γ/2 = 0.1, pH 4.2; □, 8 M urea, Γ/2 = 0.03, pH 3.2; ■, 97% formic acid; ○, methanol, 0.01 M HCl; ●, ethylene glycol, 0.01 M HCl.

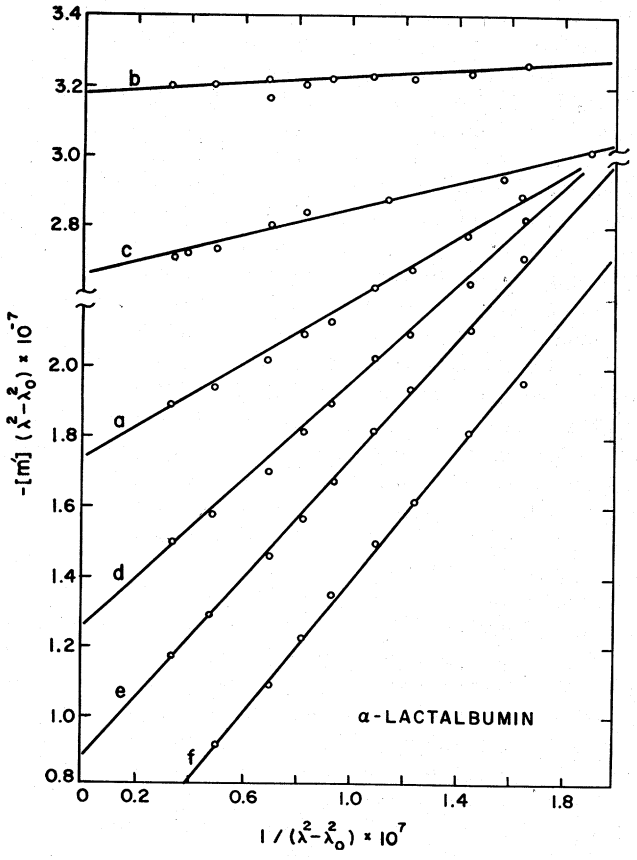

FIG. 2. Moffitt-Yang plots of native and solvent-denatured α-lactalbumin. *Curve a*, water, Γ/2 = 0.03, pH 7.0; *Curve b*, 8 M urea, Γ/2 = 0.03, pH 7.4; *Curve c*, 97% formic acid; *Curve d*, ethylene glycol, 0.01 M HCl; *Curve e*, methanol, 0.01 M HCl; *Curve f*, 2-chloroethanol.

RESULTS AND DISCUSSION

1. Interpretation of Rotatory Dispersion Data—The optical rotatory properties of the proteins studied in this paper have been interpreted by means of the Moffitt-Yang equation (24),

$$[m'] = 3M_0/100(n^2 + 2)[\alpha]_\lambda$$
$$= a_0\lambda_0^2/(\lambda^2 - \lambda_0^2) + b_0\lambda^4/(\lambda^2 - \lambda_0^2)^2 \quad (1)$$

and the one-term Drude equation,

$$[\alpha]_\lambda = A/(\lambda^2 - \lambda_c^2) \quad (2)$$

The parameters a_0 and b_0 in Equation 1 are calculated from the intercepts and slopes of $[m']$ *versus* $(\lambda^2 - \lambda_0^2)^{-1}$ plots, with λ_0 taken as 212 mμ. The constants A and λ_c in Equation 2 are similarly determined from the intercepts and slopes of $[\alpha]_\lambda \cdot \lambda^2$ *versus* $[\alpha]_\lambda$ plots (Fig. 1). The following mean residue molecular weights, M_0, were employed: β-lactoglobulin = 112, α-lactalbumin = 123.4 α_s-casein = 118, lysozyme = 111, ribonuclease = 110, and insulin = 112 (25–27). The water, 8 M urea, 2-chloroethanol, and formic acid data were corrected for the dispersion of the refractive index.[2]

The dispersion properties of methanol and ethylene glycol have not been reported, but since the effect of this correction on the a_0 and b_0 parameters of the former solvents is only of the order of 1 to 2%, no corrections were applied to the data obtained in the latter solvents.

An estimate of the helical content of any given protein can be obtained by comparing the experimentally determined b_0 value to reference values representing the contribution to b_0 of a hypothetical 100% α-helical structure. The most serious assumption made in such a comparison is that other structural features present in proteins do not contribute materially to the b_0 parameter. The commonly employed reference value for $b_0^H = -630$ (1–3) seems to predict correctly the helical content of sperm whale myoglobin (2, 28), the structure of which in the solid state has been established by x-ray crystallographic techniques (29). As a result, this b_0^H value may serve as a useful index of helicity of proteins despite the above assumptions and uncertainties concerning the precise value and meaning of this parameter (2).[3]

The rotatory dispersion constants of the various proteins in aqueous solutions and organic solvents employed in this study are given in Table I. Typical dispersion plots on which this data are based are shown in Figs. 1 to 4. It should be noted that the behavior of most proteins in helix-forming organic solvents cannot be adequately described by the one-term Drude equation (1). This so-called complex or anomalous dispersion

[2] J. G. Foss and J. A. Schellman, unpublished results. We wish to thank Dr. John G. Foss for kindly supplying us with their manuscript containing the appropriate data, prior to publication.

[3] Cassim and Taylor (30) have recently reported on an extensive study of the rotatory dispersion behavior of poly-γ-benzyl-L-glutamate in a large number of organic solvents and solvent mixtures. Their conclusion, based on measurements in 55 solvents of varying refractive index, is that instead of being largely independent of solvent, as has been widely believed (1–3), the dispersion parameter, b_0^H, varies linearly with the refractive index of the solvent. The b_0^H value extrapolates to −730° in water, or rather, in a solvent which has the refractive index of water. Since it has been reported that in a number of the solvents employed, poly-γ-benzyl-L-glutamate exists as a 3_{10}-helix rather than in an α-helix (31, 32), it is not easy to rationalize the above findings.

behavior is characterized by curvature of the dispersion plots as seen in Fig. 1.

2. Milk Proteins—The rotatory dispersion parameters of the principal milk proteins, β-lactoglobulin, α-lactalbumin, and α_s-casein, are compared in Table I. Judging from the b_0 parameters, the helical content of β-lactoglobulin appears to be very low or nearly zero (6, 21, 33), while α-lactalbumin contains a fair amount of α-helix. As a class, the caseins belong to the nonhelical category of proteins (34). The rotatory dispersion

TABLE I

*Optical rotatory properties of milk proteins and other proteins in aqueous and organic solvents**

Protein and solvent	$-A \cdot 10^{-6}$	λ_c	a_0	b_0
		mμ		
α-Lactalbumin				
Water, Γ/2 = 0.03, pH 7.0	21.0	256	−355	−235
8 M urea, Γ/2 = 0.03, pH 7.4	33.0	222	−710	−10
Formic acid	27.5	231	−590	−95
Ethylene glycol, 0.01 M HCl	15.0	268	−280	−345
Methanol, 0.01 M HCl			−180	−450
2-Chloroethanol			−105	−495
β-Lactoglobulin				
Water, Γ/2 = 0.1, pH 5.3	8.2	248	−155	−75
8 M urea, Γ/2 = 0.03, pH 3.2	36.7	221	−700	−20
Formic acid	27.0	229	−530	−90
Ethylene glycol, 0.01 M HCl			−85	−420
Methanol, 0.01 M HCl			+10	−505
2-Chloroethanol			+25	−455
α_s-Casein				
Water, Γ/2 = 0.03, pH 7.4	25.0	223	−530	−45
8 M urea, Γ/2 = 0.03, pH 3.3	30.5	217	−620	0
Ethylene glycol	15.0	248	−295	−150
Methanol, 0.01 M HCl			−55	−355
2-Chloroethanol			+5	−300
Lysozyme				
Water, pH 5.3	16.0	242	−295	−150
Water, Γ/2 = 0.1, pH 7.1	15.0	243	−275	−145
8 M urea, Γ/2 = 0.1, pH 3.2	28.8	212	−530	−10
Ethylene glycol, 0.01 M HCl	10.5	266	−190	−225
2-Chloroethanol			+10	−350
Bovine serum albumin				
Water, Γ/2 = 0.1, pH 5.4	17.0	259	−340	−310
8 M urea, Γ/2 = 0.1, pH 3.6	33.2	222	−560	−40
Formic acid	25.5	235	−530	−75
Ethylene glycol, 0.01 M HCl			−265	−360
Methanol, 0.01 M HCl			−200	−425
2-Chloroethanol			−85	−420
Ribonuclease				
Water, Γ/2 = 0.03, pH 3.4	21.5	232	−415	−95
8 M urea, Γ/2 = 0.03, pH 3.5	31.0	213	−575	0
Ethylene glycol, 0.01 M HCl	15.0	248	−245	−115
2-Chloroethanol			−100	−385
Insulin				
Water,† Γ/2 = 0.1, pH 1.9	7.8	264	−145	−200
Water, Γ/2 = 0.1, pH 2.3	8.3	266	−150	−200
8 M urea, Γ/2 = 0.1, pH 3.5	27.0	226	−510	−60
Ethylene glycol, 0.01 M HCl	17.0	242	−310	−135
Methanol, 0.01 M HCl	14.0	256	−255	−240
2-Chloroethanol			−160	−280

* a_0 and b_0 values are reported to the nearest ±5°. Where A and λ_c are not given, the rotatory dispersion is anomalous (Fig. 1).

† Mann Research Laboratories sample (Lot K-2459); the rest of the insulin data refer to an Eli Lilly sample (Lot PJ-4086).

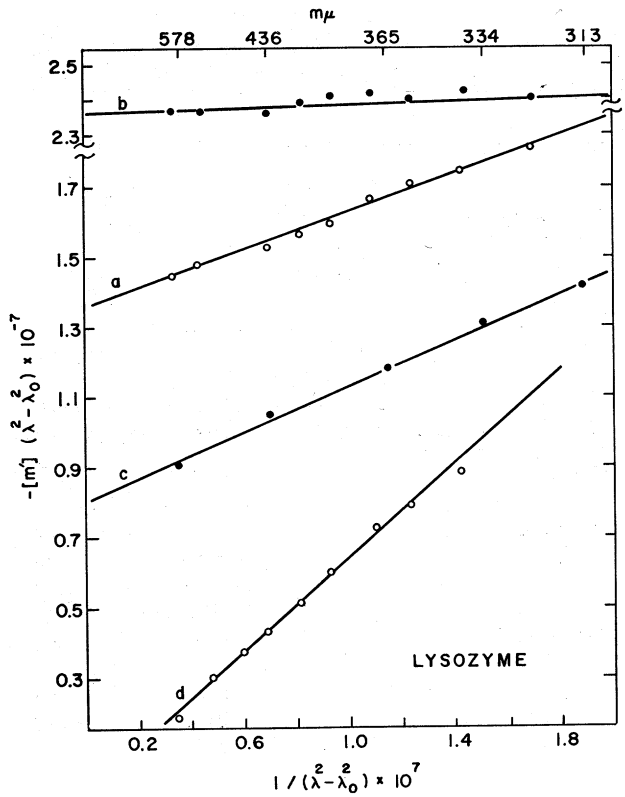

FIG. 3. Moffitt-Yang plots of lysozyme in aqueous and organic solvents. *Curve a*, water, pH 5.3; *Curve b*, 8 M urea, Γ/2 = 0.1, pH 3.2; *Curve c*, ethylene glycol (after 24 hours at 25°); *Curve d*, 2-chloroethanol.

properties of the caseins in aqueous solutions are similar to the properties of other proteins in random coil-forming solvents. This has been interpreted to mean that in neutral salt solutions the caseins have a disorganized randomly coiled structure (34). For example, α_s-casein (23), has the following parameters: b_0 = −45, a_0 = −530, and λ_c = 223; this should be compared with b_0 = 0 to −95, a_0 = −510 to −710, and λ_c = 212 to 235 obtained with the globular proteins of this study in 8 M urea and formic acid. α_s-Casein, interestingly, possesses no space-confining disulfide bridges; it has, however, a fairly high content of proline which is known to interfere with helix formation (9% as compared with the usual 2 to 5% proline in other proteins). As a result, it is not surprising that even under conditions favoring helix formation, that is, in 2-chloroethanol and acidic methanol, the amount of helical structure present is relatively low.

3. Bovine Serum Albumin—The helical content of the serum albumins is in the neighborhood of 50% (21, 35, 36). As has been noted (23), this fact and the presence of a fairly large number of cross-link-forming cystine groups will tend to limit the possibility of further helix formation in organic solvents. The data of Table I indicate that the b_0 values are only slightly increased in acidic ethylene glycol. Even under conditions of maximal helix formation, the change in b_0 is of the order of 10 to 15%, yet the conformational readjustment in glycol, methanol, or 2-chloroethanol must be fairly pronounced, since this process is accompanied by the exposure of more than half of the buried tyrosyl side chains (23).

4. Lysozyme—The conformation of lysozyme remains largely unaffected in ethylene glycol media, indicating that it is a fairly

TABLE II

Effect of time on optical rotatory dispersion parameters of salt-free lysozyme in ethylene glycol at 25°

Time	a_0	b_0
hrs		
1	−176	−199
3½	−178	−170
7	−178	−154
24	−178	−165
48	−178	−159

TABLE III

Effect of electrolyte and HCl on optical rotatory parameters of α-lactalbumin, lysozyme, and ribonuclease in ethylene glycol

Protein and added electrolyte	a_0	b_0
α-Lactalbumin		
0.2 M KCl	−200	−240
0.2 M LiCl	−170	−290
0.01 M HCl	−280	−345
0.02 M HCl	−245	−360
Lysozyme*		
None	−180	−160
0.2 M KCl	−105	−145
0.01 M HCl	−190	−225
Ribonuclease		
0.2 M KCl	−175	−90
0.2 M LiCl	−155	−130
0.01 M HCl	−245	−115
0.02 M HCl, 0.08 M LiCl	−195	−140

* After standing for 5 to 24 hours at 25° (see Table II).

stable protein. Jirgensons (37) has found that neither the addition of detergent to aqueous solutions of this enzyme nor prolonged heating at 50° in the presence of detergent had a very significant effect on its rotatory properties. The slight time dependence of b_0 in electrolyte-free glycol solutions, which has not been observed with the other proteins in this study, indicates that a slight conformational change does occur in this solvent (Table II). This change is also reflected in the slight increase of the difference spectral parameter R_M (23).

In a recent series of papers Shechter and Blout (21, 22) and Shechter, Carver, and Blout (35) have advanced a new analysis of the rotatory dispersion of proteins and polypeptides, based on the use of a modified two-term Drude equation. It is gratifying that their estimates of the helix content of a number of proteins (with poly-α-L-glutamic acid in the helical and random conformations as references) are not appreciably different from the estimates obtained by use of the Moffitt equation (35). Thus, for lysozyme and ribonuclease in 2-chloroethanol their analysis gave estimates of helix content of 48 and 64%. Our corresponding values, based on the Moffitt equation, with the reference value of b_0^H taken as −630, are 56 and 61%, respectively (Table I).

5. Ribonuclease and Insulin—Sage and Singer (5) have found that the rotatory properties of ribonuclease are largely unaffected in ethylene glycol-0.2 M KCl. The conformation of this enzyme is altered in this solvent, since there is a change in the ease of ionization and in the location of the buried tyrosyls (23). Ribonuclease dissolved more readily in ethylene glycol in the presence of 0.01 M HCl than in the presence of 0.2 M KCl or LiCl. The latter solutions had to be clarified by centrifugation before rotatory dispersion measurements could be made, suggesting that ribonuclease is more aggregated in the presence of neutral electrolyte than in acid. The data of Table III indicate that the nature of the electrolyte has a small effect on the rotatory properties of ribonuclease in glycol media. It should be also noted that electrolyte has an appreciably greater effect on a more easily deformable protein such as α-lactalbumin. This is fairly apparent from the changes in the rotatory dispersion parameter, b_0, given in Table III, as well as the changes in the difference spectral parameter, R_M, which is a measure of the fraction of chromophoric amino acids exposed in a given protein (23). It appears that the fraction of exposed tyrosyl residues in ribonuclease and tryptophyl residues in lysozyme is largely unaffected in glycol media containing acid or KCl. With α-lactalbumin, on the other hand, the extent of unfolding and exposure of chromophoric groups in this solvent can be changed quite considerably by a change in acid or electrolyte content of the solvent medium.

Insulin is more readily deformable in ethylene glycol than is ribonuclease. One of the interesting findings of the previous paper was that the fraction of tyrosyl residues exposed in this solvent was approximately the same as in acidic methanol and 2-chloroethanol. In fact, as far as one can judge from the change in R_M (23), the extent of unfolding of insulin in this solvent seems to be even greater than in 8 M urea. The shift in b_0 to a less negative value indicates that the unfolding process in this solvent is accompanied by a net loss of right-handed α-helical folding in the polypeptide chains.

What is unusual about insulin, when compared with the behavior of other proteins (Table I), is the fact that even in solvents that strongly favor helix formation, the change in b_0 is relatively small. It has been suggested that insulin may possess helical regions of both right- and left-handed sense (2, 38). This simple hypothesis would nicely explain the relatively high b_0 values of insulin in acidic methanol and 2-chloroethanol. Unfortunately, it is difficult to see why the intrinsically less stable left-handed helical regions[4] would be preserved in ethylene glycol, while some of the right-handed regions were being destroyed (*i.e.* b_0 becomes less negative). Moreover, while there is only a marginal change in b_0 in methanol, the exposure of a substantial fraction of the previously buried tyrosyls indicates that the native structure is substantially altered in this solvent.

What are the reasons for the anomalous rotatory power of insulin in these solvents, and what are the structural bases which would explain the observed rotatory properties of this protein? Two factors need to be considered when the rotatory properties of helical regions in proteins are examined: (*a*) the relative stability of short helical regions, and (*b*) the effect of chain length on the rotatory power of such helices (*i.e.* end effects). In this relation, it should be noted that the steric constraints imposed by the three cystine cross-links and the single proline

[4] Huggins (39) (see also Reference 40) has made the fundamental observation some time ago that the formation of left-handed helices from L-amino acids is impeded by interference of the β carbon atom and a carbonyl oxygen in the next turn of the helix. No steric difficulties were, however, encountered in the construction of right-handed helices from models of poly-γ-methyl or poly-γ-benzyl-L-glutamates (39, 40).

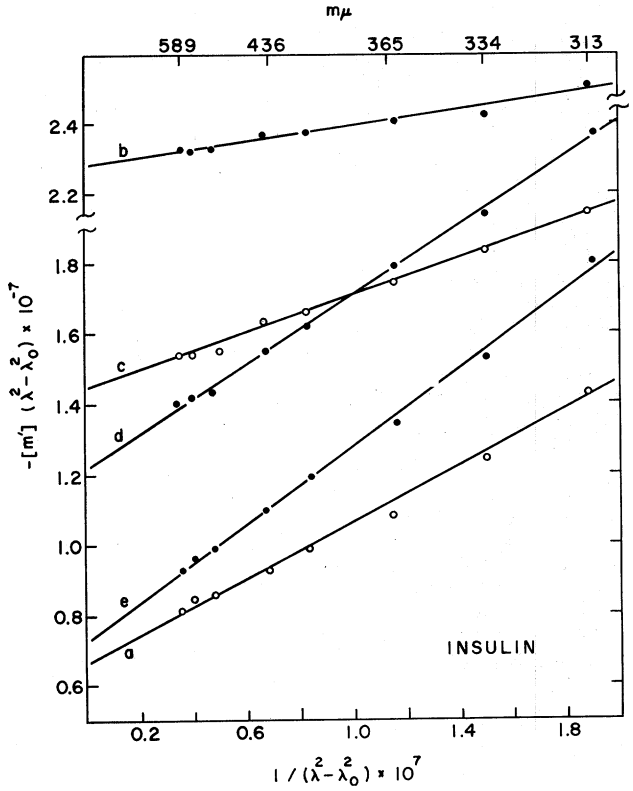

FIG. 4. Moffitt-Yang plots of insulin in aqueous and organic solvents. *Curve a*, water, $\Gamma/2 = 0.1$, pH 2.3; *Curve b*, 8 M urea, $\Gamma/2 = 0.1$, pH 3.5; *Curve c*, ethylene glycol, 0.01 M HCl; *Curve d*, methanol, 0.01 M HCl; *Curve e*, 2-chloroethanol.

residue in insulin (41) will only allow the formation of fairly short helical regions, *i.e.* segments consisting of 5 and 8 amino acids in the A chain and 6, 11, and 8 in the B chain. Moreover, one has no assurance that even in strongly helix-promoting solvents, the shorter polypeptide segments will be sufficiently stabilized so as to assume a fully helical conformation.[5] In relation to item *b* the studies of Mitchell, Woodward, and Doty (42) and Goodman *et al.* (43, 44) on the rotatory properties of low molecular weight polypeptides in helix-forming solvents and the more recent calculations of Woody (45) (see also Reference 46) on the influence of end effects on the rotatory power of helices of finite length should be cited. The results of these workers suggest that end effects may reduce the b_0 values of the shorter helical segments in insulin by as much as 50%. This could then explain why the apparent changes in α-helical content, as judged by the changes in b_0, are only about 5 to 15%, in going from water to acidic methanol and 2-chloroethanol.

In view of the fact that both the instability of short helices and the effects of end groups will lower the absolute value of b_0, it is not possible to make an unequivocal decision concerning the location of helical and nonhelical regions based only on the known amino acid sequence and experimentally determined b_0 parameters. It is, however, apparent that one is not forced to postulate the existence of helices of both left-handed and right-handed screw sense to explain the rotatory properties of insulin.

[5] The x-ray crystallographic studies of Kendrew *et al.* (29) indicate that the shortest helical segments in sperm whale myoglobin consist of seven to nine amino acid residues.

SUMMARY

The optical rotatory properties of a number of proteins are examined in two helix-promoting solvents, 2-chloroethanol and acidic methanol, and two random coil-forming solvents, 8 M urea and formic acid, and compared with their rotatory dispersion properties in the native state in aqueous media. In addition, their rotatory properties in ethylene glycol containing neutral electrolyte or acid are reported.

Three of the seven proteins examined, α-lactalbumin, bovine serum albumin, and insulin, are fairly helical proteins, while the other four, α_s-casein, β-lactoglobulin, ribonuclease, and lysozyme, are largely nonhelical proteins. The apparent helical content of ribonuclease and lysozyme (estimated from the changes in the Moffitt parameter b_0) is essentially unaffected by ethylene glycol. Difference spectral studies of the accompanying paper indicate the same, although ribonuclease is found to be partly unfolded in this solvent. The denaturation of insulin in ethylene glycol is accompanied by a net decrease in helical content; in the other proteins examined there is an increase in helical folding.

The change in helical content of bovine serum albumin, α-lactalbumin, α_s-casein, and β-lactoglobulin under maximal conditions of helix formation is about 20 to 70%. However, even under these conditions the full helical development of the various proteins studied is not attained.

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